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Short Communication

Characterization of the First *Batrachochytrium dendrobatidis* Isolate from the Colombian Andes, an Amphibian Biodiversity Hotspot

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Abstract: The pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), constitutes a significant threat to more than 790 amphibian species occurring in Colombia. To date there is no molecular or morphological description of strains infecting Colombian populations. Here we report the genetic and morphological characterization of the first Colombian isolate of *Bd* (strain EV001). Our goals were threefold: (1) to characterize the morphology of EV001 using light and scanning electron microscopy, (2) to genotype this strain by direct sequencing of 17 polymorphic nuclear markers developed previously, and (3) to compare our findings with published reports on strains from other areas of the globe. We found that EV001 is morphologically consistent with previously described strains. Multi-locus genotyping suggested that EV001 is grouped genetically with Panamanian strains and is most similar to strain JEL203 isolated from a captive individual. This finding fills an important gap in our knowledge of Neotropical strains of *Bd* and provides a baseline for further evolutionary and functional analyses.

Keywords: Chytridiomycota, Colombia, Andes, multi-locus genotyping, morphological description

Many amphibian populations around the world have declined drastically in the last 40 years (Wake and Vredenburg 2008). One of the main drivers of amphibian declines is chytridiomycosis, an emergent disease caused by the chytrid fungus, *Batrachochytrium dendrobatidis* (hereafter, *Bd*), the only known Chytridiomycota capable of causing disease in vertebrates (Longcore et al. 1999). This pathogen infects and causes population declines in a wide variety of amphibian species (e.g., Lips et al. 2006; Crawford et al. 2010). *Bd* reproduces via zoosporangia within epidermal cells of its amphibian host, leading to hyperkeratosis and the disruption of electrolyte balance (Voyles et al. 2009). Some amphibians appear to be resistant to the disease in the face of infection, yet the mechanisms underlying resistance are not well understood. Skin microbiota and anti-microbial peptides could play a key role in defense against the lethal effects of the pathogen (Woodhams et al. 2007; Harris et al. 2009; Becker and Harris 2010).

Two hypotheses have been proposed to explain the chytridiomycosis epizootic. The 'Endemic Pathogen Hypothesis' suggests that *Bd* naturally occurs on most continents, yet only recently has it become pathogenic for

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amphibians due to possible environmental triggers (Rachowicz et al. 2005). The 'Novel Pathogen Hypothesis', in contrast, states that *Bd* is a newly emerging pathogen that spread recently across the globe (Rachowicz et al. 2005; Skerratt et al. 2007), and finds support in numerous studies comparing *Bd* from different continents through multilocus sequence typing (MLST; Morehouse et al. 2003; Morgan et al. 2007; James et al. 2009). A complete understanding of the spatial and temporal diversification of this pathogen, however, will require more thorough sampling including the morphological, functional, and molecular characterization of new isolates.

Central and South America together host half of the world's amphibian species, many of which have suffered population declines over the last 30 years (Young et al. 2001). Temporal and spatial patterns of amphibian declines suggest that *Bd* may have arrived in South America in the early 1980s via at least three independent colonization events (Lips et al. 2008): Andean Ecuador (Ron et al. 2003), Andean Venezuela (Bonaccorso et al. 2003), and southeastern Brazil (Heyer et al. 1988; Carnaval et al. 2006). From these three hypothesized points of entry, *Bd* is thought to have radiated outward, entering Colombia from the east via Venezuela and/or from the south via Ecuador (Lips et al. 2008).

Colombia hosts the second most diverse amphibian fauna in the world, with about 790 described species (Ruiz-Carranza et al. 1996; AmphibiaWeb 2012), yet limited information exists on the current conservation status of most amphibian species (Rueda-Almonacid et al. 2004), possible amphibian die-offs (Lynch and Grant 1998) or the distribution and diversity of *Bd*. Chytrid infection has been reported in frogs from two sites in the eastern (Ruíz and Rueda-Almonacid 2008) and various sites in the western chain of the Colombian Andes (Velásquez-Escobar et al. 2008; Urbina and Galeano 2011), as well as on the Pacific island of Gorgona (Flechas et al. 2012). To date, the oldest known record of *Bd* in Colombia comes from a frog collected in 1994 roughly 300 km north of Ecuador at Cerro El Inglés, Valle del Cauca (Velásquez-Escobar et al. 2008). As a first step towards understanding the functional and genetic diversity of *Bd* and its evolutionary history in Colombia, we undertook the following morphological and genetic characterization of the first strain of *Bd* (EV001) collected and isolated in Colombia.

EV001 was isolated from the skin of a juvenile of *Rheobates palmatus* (Werner 1899), a dendrobatoid frog (Grant et al. 2006) that occurs between 350 and 2400 meters above sea level (masl) in the eastern and central Andes of Colombia. This species is associated mainly with streams but appears to be widely tolerant of habitat disturbance and can be found breeding in man-made pools (Lüddecke 2003). Individuals of *R. palmatus* were collected near Ubaque, Departamento de Cundinamarca (04°26′12″N, 73°55′10″W, at 1950 masl), 56 km east of Bogotá. To determine whether frogs were infected with *Bd*, we performed end-point PCR from cotton swabs following Annis et al. (2004) with slight modifications. The amplification protocol consisted of an initial denaturation at 93°C for 2 min, followed by 35 cycles of 45 s at 95°C, 45 s at 55°C and 1 min at 72°C, with a final extension at 72°C for 10 min. The amplified fragments were separated by electrophoresis using 1% agarose gels.

To isolate *Bd*, we euthanized infected frogs by pithing, removed skin samples from the venter, hind limbs, and the ventral surface of hands and feet, and cut them into 2 × 2 mm squares. Each piece of skin was cleaned in water-based agar with antibiotics in order to remove bacteria, yeast, and fungal spores. After thoroughly cleaning the skin samples, we transferred them to a fresh plate with TGh media (10 g tryptone, 10 g agar, 4 g gelatine hydrolysate, 1000 mL distilled water) following a protocol described by J. E. Longcore (pers. comm.). Plates were sealed and incubated at 23°C until *Bd* growth was detected. Active *Bd* cultures were then cryopreserved following the protocol described in Boyle et al. (2003).

Zoospores were measured using scanning electron microscopy (JEOL JSM-6490LV) from pure EV001 cultures in TGh media after direct critical point drying (SAMDRI®-795) and metallization (Dentom Vacuum Desk IV). To estimate the size of zoosporangia, we used light microscopy under 100× magnification on 7-day-old pure TGh agar cultures. The diameter of sporangia was estimated as the length of the longest axis. Digital measurements were taken using the software ImageJ v.1.44 m (Abramoff et al. 2004). Sporangia showing at least one discharge papilla averaged 25.97 µm (SD = 3.91 µm; $N = 60$) in length and zoospores averaged 3.14 µm (SD = 0.33 µm; $N = 10$) in diameter (Fig. 1).

Genomic DNA was extracted from axenic cultures using GeneReleaser (BioVentures, Murfreesboro, TN) following manufacturer's protocols. To perform multi-locus genotyping of EV001 we used 17 marker loci as follows: APRT13, BdC5, BdC18.1, BdC18.2, BdC24, CTSYN1, R6046, 6164, 9893, mb-b13, b7-10c, 6677X2, 6873X2, 8009X2, 8329X2, 8392X2, 8702X2 (Morehouse et al. 2003; James et al. 2009). PCR products were cleaned and directly

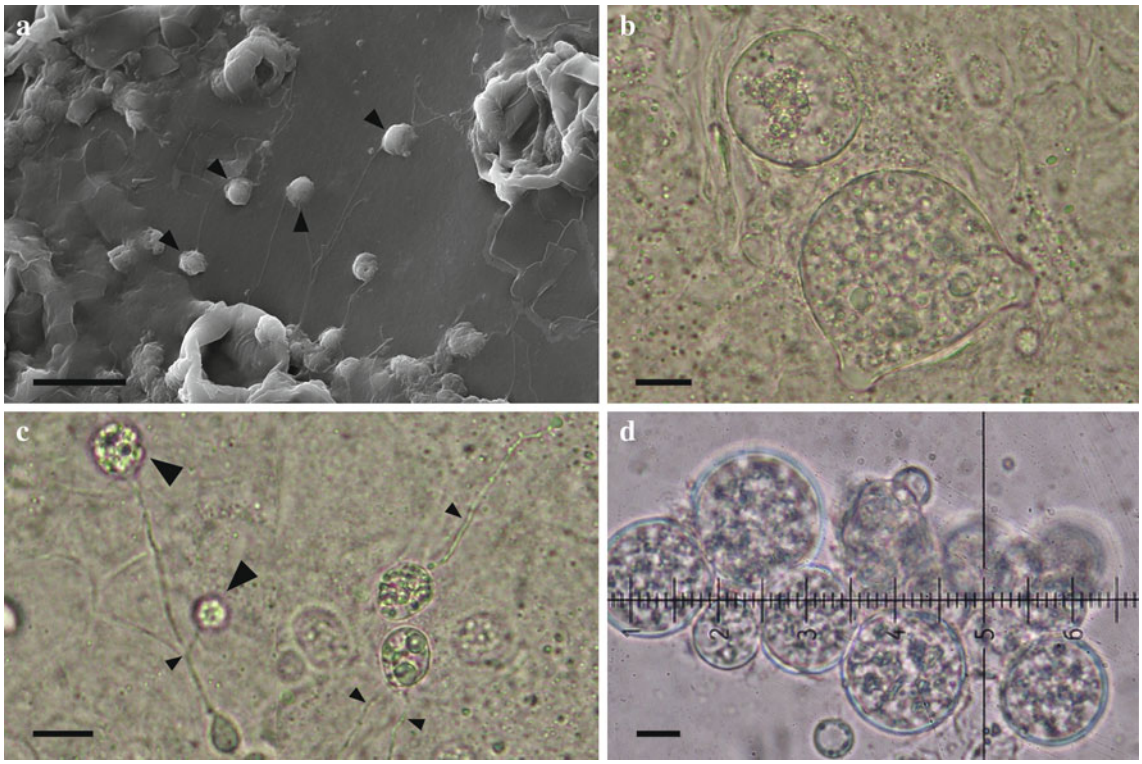

Figure 1. Morphological characteristics of *Batrachochytrium dendrobatidis* (*Bd*) strain EV001. **a** Scanning electron micrograph of zoospores (*black arrowheads*). **b** Thalli of *Bd* in amphibian host tissue. **c** Different stages of development of the thallus. Note the spread of long rhizoids (*small arrowheads*) and the emergence of multiple zoosporangia (*large arrowheads*) from a single thallus. **d** Zoosporangia in TGh media. Photos **b** and **c** correspond to skin directly removed from an individual. The photos were taken within 2 h after the frog was euthanized. Integers on the scale bar correspond to units of 10 μm.

sequenced with forward and reverse primers using Sanger sequencing technology. Heterozygous nucleotides at diagnostic sites were identified by double peaks in both directions observable in electropherograms (Hare and Palumbi 1999). Diploid sequences heterozygous for diagnostic indels were identified by the presence of stutter in both electropherograms following the indel (i.e., on opposite sides in forward and reverse reads). As an internal quality control for the genetic data, we also genotyped the *Bd* strain JEL423, and compared our results with published data obtained from these same 17 markers (James et al. 2009). DNA sequences for EV001 and the re-genotyped JEL423 strains were aligned manually against the published data. At each locus the two new diploid sequences matched known genotypes and were assigned letter codes, 'a' or 'c' for homozygotes and 'b' for fixed heterozygotes, following James et al. (2009). Our re-genotyping of JEL423 matched the published data (James et al. 2009) at 16 of 17 loci. According to James et al. (2009), JEL423 should have genotype 'b' at marker 'mb-b13' whereas we obtained genotype 'c' for this strain, as well as for EV001. Both of our strains also showed one extra base near the 5′ end of the fragment. One hypothesis that could explain both of these observations would be that we amplified a marker similar but paralogous to 'mb-b13,' and therefore coded this marker as 'missing data' for our analyses to be conservative in our estimation of genetic differences. Combining all data into a matrix with 17 characters and 61 *Bd* isolates, we used *PAUP** (Swofford 2000) version 4a123 to construct a neighbor-joining (NJ) tree (Saitou and Nei 1987) based on mean distances, using the minimum evolution criterion and breaking ties randomly (Fig. 2). Statistical support for nodes was assessed via non-parametric bootstrap using 2,000 re-sampling replicates (Felsenstein 1985). The multi-locus genotype dataset was submitted to http://DataDryad.org, under reference number doi:10.5061/dryad.1b68v.

Compared to previously genotyped strains, the Colombian isolate EV001 showed genetic differences at three of 17 loci examined. In the NJ analysis EV001 clustered with an Australian strain, a captive strain, and a set of identical Panamanian isolates (Fig. 2). The phenotypic characteristics observed in EV001 are in accordance with the morphometric parameters previously described for *Bd*,

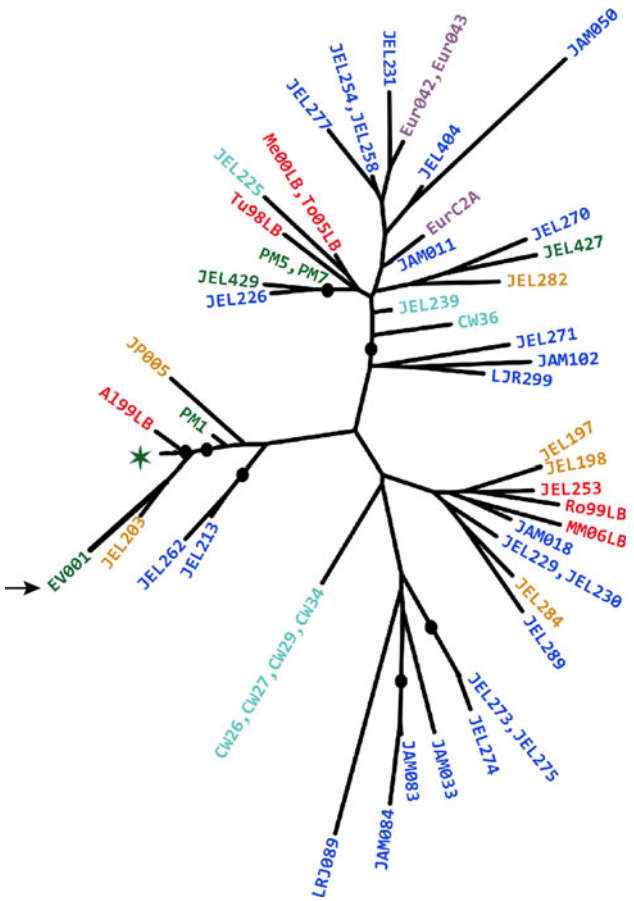

Figure 2. Unrooted minimum evolution neighbor-joining tree based on mean distances of 61 strains, including 59 published genotypes (James et al. 2009) plus sequencing of an additional sample of the strain JEL423 and the isolate EV001, reported here for the first time (see *black arrow* in the far *left*). Tree inferred from 17 polymorphic nuclear markers, each with three possible states (James et al. 2009). *Star symbol* represents the position of strains JEL408, JEL409, JEL415, JEL424, JEL425 and the original plus newly genotyped samples of JEL423. *Black dots* indicate internal nodes with >50% bootstrap support, none of which scored >80% (branches leading to identical genotypes were counted as external). *Colored* strain labels indicate geographic origin, following James et al. (2009): *green* Neotropics, *blue* North America, *red* Australia, *orange* captive animals, *purple* Europe, and *teal* Africa.

in terms of size of zoosporangia (Longcore et al. 1999; Farrer et al. 2011). Zoosporangium diameter ranges from roughly 15–65 µm among 'global panzootic lineage' isolates (*Bd*-GPL; Farrer et al. 2011), for example, and our mean size estimate of 26 µm is close to the median of approximately 28 µm. Since the Panamanian strain JEL423 is classified as a member of the *Bd*-GPL (Farrer et al. 2011; Schloegel et al. 2012) and EV001 appears to be very closely related to JEL423 (Fig. 2), we infer that the Colombian strain is also a member of the *Bd*-GPL. Given that EV001 is embedded within *Bd*-GPL, which itself is sister to and well diverged from a second lineage present in South America, *Bd*-Brazil (Schloegel et al. 2012), we conclude that EV001 is not related to *Bd*-Brazil.

While the genetic similarity to the Panamanian isolates might suggest a historical demographic connection between Colombian and Panamanian *Bd*, the NJ tree as a whole shows little correlation between geography and genetic similarity (James et al. 2009). Although our NJ analysis of 17 loci may be insufficient to resolve the geographic origins of Colombian *Bd*, further samples from this region will be crucial to understanding the origin and possible spread of *Bd* (Lips et al. 2008). More genetic markers and more isolates from different regions of Colombia may be required to determine how many colonization events were involved and from what source populations *Bd* may have arrived in Colombia.

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